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RESEARCH PAPER

Preparation and characterization of PCL polymeric scaffolds coated with chitosan/ bioactive glass/gelatin nanoparticles using the tips methodology for bone tissue engineering

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ABSTRACT

Objective(s): The present study aimed to prepare polycaprolactone (PCL) scaffolds with high porosity and pore interconnectivity, in order to copy the microstructure of natural bones using the thermally induced phase separation (TIPS) technique.

Materials and Methods: The scaffolds were coated with chitosan (CH), bioactive glass (BG), and gelatin nanoparticles (GEL NPs) and assessed using scanning electron microscopy and Fourier-transform infrared spectroscopy (FTIR). The effects of various coatings on the scaffold characterizations, proliferation, and mineralization of MG-63 osteoblast-like cells were evaluated.

Results: The size of the prepared BG and GEL NPs was estimated to be 400 and 234 nanometers, respectively. The porosity and contact angle of PCL/CH/GEL NPs/BG was 74% and 72°, respectively. Weight loss and electron microscopy evaluations indicated the improved degradation rate of the scaffolds and spreading tendency of the cells on the scaffolds when modified as compared to the scaffolds that were purely obtained from PCL. In addition, the in-vitro studies revealed that the MG-63 cells cultured on the PCL/CH/GEL NPs/BG scaffolds showed improved cell proliferation more significantly compared to the scaffolds obtained from PCL, PCL/CH/GEL NPs, PCL/CH, and PCL/GEL NPs. Mechanical examinations also showed that PCL/CH/GEL/BG scaffolds had the highest mechanical strength compared to other groups (i.e., 4.66 Mpa). Cell viability was estimated to be 96.7%, and the alizarin red test indicated the significant improvement of mineralization in the PCL/CH/GEL NP group.

Conclusion: According to the results, the PCL scaffolds that were modified by CH/GEL NPs/BG had the high potency to be used as bone tissue engineering scaffolds.

Keywords: *Bioactive Glass, Chitosan, Gelatin NPs, PCL, Thermally Induced Phase Separation*

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INTRODUCTION

Tissue engineering provides methods to combine biodegradable and biocompatible scaffolds with cells and bioactive agents in order to replace defective and damaged tissues [1]. An essential requirement for a scaffold, particularly in bone tissue engineering, is the proper and well-regulated interconnected porosity, so that cells could grow with appropriate physical shapes, while assisting the vascularization of primary tissues [2, 3]. Thermally induced phase separation (TIPS) is often applied to fabricate such a structure [4]. In this technique, a homogeneous multicomponent system becomes thermodynamically unstable under defined circumstances so as to be divided

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into two distinct phases [5]. Following that, the dissolved polymer is observed in a polymer-rich phase and polymer-lean phase. After the removal of the solvent, the polymer-rich phase is converted into porous scaffolds with appropriate pore geometry and interconnectivity [6, 7].

Numerous natural and synthetized components are currently available for tissue engineering, including gelatin, collagen, cellulose, poly(ε-caprolactone) (PCL), poly(L-lactic acid) (PLLA), and poly(lactide-co-glycolide) (PLGA) [8, 9]. Among these methods, PCL has gained remarkable attention since it has been approved by the United States Food and Drug Administration (FDA). The polymer is flexible, biocompatible, and biodegradable, offering with proper mechanical and toxicity properties [10].

To provide scaffolds with desirable properties, scaffold coating could be used effectively and frequently [11]. For instance, the surface modification of synthetic scaffolds with PCL has been widely adopted to improve cell adhesion [12, 13].

This technology has been widely applied in tissue engineering owing to its interesting properties, such as biological origin, biodegradability, biocompatibility, cost-efficiency, and availability.

Chitosan (CH) is a biocompatible and biodegradable polysaccharide [14], and gelatin (GEL) is an economical biodegradable protein with wide applications in food, cosmetics, and pharmaceutical industries [15, 16]. These compounds have a wide range of applications in tissue engineering. GEL has notable features of collagen without its immunogenicity and pathogen transmission potency [17].

Recently, bioactive glasses (BGs) have been utilized as glass-ceramic biomaterials with surface reactivity and investigated in bone repairing scaffolds [18]. These compounds play a key role in the binding of the scaffold with the surrounding tissues. Furthermore, they stimulate angiogenesis and release ions that activate the expression of osteogenic genes [19].

The present study aimed to fabricate porous PCL scaffolds coated with GEL nanoparticles (NPs), CH, and BG using the TIPS method and evaluate the effects of various coatings on the attachment, morphology, proliferation, and mineralization of MG-63 osteoblast-like cells. To the best of our knowledge, no prior studies have been focused on the effects of these coatings on PCL scaffolds.

MATERIALS AND METHODS

In this study, PCL (molecular weight: 80,000-90,000 g/mol, Sigma Aldrich, USA), GEL (type B, obtained from bovine skin, Sigma Aldrich, USA), sodium hydroxide, CH (molecular weight: 7 kD, degree of deacetylation≥80%, Zheugzhou Sigma, China), and BG 45S5 (Mod Zist Samaneh Pishro Co., Iran) were used for the preparation of the composites. The MG-63 cell line was provided by Pasteur institute of Iran (Tehran, Iran). In addition, Dulbecco's modified eagle medium/nutrient F-12 ham (DMEM/F12) and fetal bovine serum (FBS) were purchased from Gibco (USA). Penicillin, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), alizarin red staining solution (pH: 4.2), 1,4-dioxane, and acetic acid were purchased from Sigma Aldrich (USA). All the chemicals were of an analytical grade and used as received without further purification.

Preparation of GEL NPs using the desolvation technique

In this study, GEL NPs were selected for their biocompatibility, biodegradability, ability to increase cell cohesion and cell reaction, and hydrophilic nature. Initially, 50 milligrams of GEL was dissolved in five ml of distilled water on a stirrer. Following that, the pH of the solution was set at less than 6.0 (i.e., isoelectric point) using acetic acid, and the solution was quickly injected to 15 ml of ethanol at the temperature of 50˚C and mixed to form GEL NPs.

Preparation of PCL scaffolds using the TIPS technique

At this stage, PCL was dissolved in 1,4-dioxane (5% w/v) for four hours, and the solution was heated to the temperature of 60 for 30 minutes. Afterwards, the solution was quenched rapidly to -80°C and preserved for three hours. The samples were immediately transferred to a freeze dryer with the temperature of -77˚C (Christ, Pmma121550, Spain) and preserved for 48 hours in order to obtain the scaffolds.

For the surface functionalizing of PCL, the samples were initially immersed in NaOH solution (1 M) for 60 minutes so as to yield fiber surfaces bearing carboxylic groups. Afterwards, the samples were washed with distilled water three times and transferred to an oven (40˚C) to be dried.

Surface modification of the scaffolds

To coat the samples with CH/GEL NPs/BG, they

were immersed in dispersions of CH (2% w/v), BG (0.2% w/v), and/or GEL NPs for four hours (deep coating). Following that, the scaffolds were transferred to the freeze drier and preserved at the temperature of -80˚C.

The fabricated samples included PCL, PCL/CH, PCL/CH/BG, and PCL/CH/ GEL NPs, PCL/CH/GEL NPs/ BG, PCL/GEL NPs/BG, and PCL/GEL NPs.

Scanning electron microscopy of the PCL scaffolds

After coating with gold for 300 seconds using a sputter coater (model: SC7620, Emitech, UK) at the accelerating voltage of 20 kV, the morphology of the scaffolds was assessed via scanning electron microscope (SEM; AIS2100, Seron Technology, South Korea).

Fourier-transform Infrared (FTIR) Spectroscopy

Fourier-transform infrared (FTIR) spectroscopy was used to examine surface coating of the PCL scaffolds based on the PerkinElmer Spectrum GX system (USA).

Size measurement of the NPs

Particle size (d50) was measured via dynamic light scattering (DLS) using Scatteroscope I (Qudix, Korea). The samples were analyzed freshly with no dilution prior to the analysis.

Contact angle measurement

Contact angle was measured using the sessile drop method to assess the hydrophilicity of the scaffolds (G10, KRUSS, Germany).

Measurement of pH

The fabricated scaffolds were immersed in saline solution (0.9% w/v) with the temperature of 37˚C and pH of 6.37 for four weeks (final PCL concentration: 3.0% w/v). The pH of the solution was measured weekly using InoLab (WTW, Germany).

Weight loss measurement

To measure weight loss, 10 milliliters of phosphate buffer saline (PBS) was applied to immerse the scaffolds. Afterwards, the samples were extracted and dried at predetermined degradation times. Weight loss was calculated using the following equation [20]:

Weight loss (%)=(W0-W1)/W0×100

where *W0* represents the initial weight of the samples, and *W1* denotes the weight of samples when removed from the media.

Porosity assessment

The scaffolds were cut equally in order to assess porosity using the liquid displacement method. To this end, 96% ethanol was poured into a graduated cylinder, with the initial volume recorded. Following that, the scaffolds were immersed in ethanol for approximately 10 minutes, and the new volume was recorded. Subsequently, the scaffolds were removed, and the volume of ethanol was recorded after the removal of the scaffolds as well. The percentage of porosity in the samples was determined using the following equation [21]:

Porosity (%) = (V1-V3) / (V2-V3) ×100

where *V1* represents the initial volume of ethanol, *V2* is the recorded volume after the immersion of the scaffolds (ethanol filling the pores), and *V3* indicates the volume of ethanol after the removal of the scaffolds.

Determination of mechanical properties

At this stage, the compressive strength of the scaffolds was measured using a universal testing machine (Koopa, model: UV1) at the crosshead speed of 1.3 mm/min. Compressive strength (MPa) was determined using the following equation:

Compressive Strength (CS) = F/A

where *F* is the applied compressive load (Newton), and *A* shows the surface area (mm^2) of the PCL scaffolds perpendicular to the load axis (m^2).

Cell culture examination

Human cell line MG-63 was cultured on a medium containing DMEM/F12, 10% v/v FBS, streptomycin (100 mg/ml), and penicillin (100 unit/ml) in a humidified incubator at the temperature of 37°C, accompanied by 5% CO_2. The scaffolds were irradiated by UV for one hour and washed with PBS and DMEM/F12 twice. Subsequently, 5×10^3 of the third-passage cells were cultured on the scaffolds in 96-well plates. After incubation for one hour, 0.15 milliliter of the cell culture medium containing FBS was added to each well. The medium was replaced every 24 hours.

Cell proliferation and viability examinations

After 2-3 days of incubation, MTT assay was performed to investigate cell proliferation. To this end, 150 microliters of MTT (0.5 mg/ml) was added

when the media on the cells was removed from each well. Following that, the cells were incubated at the temperature of 37°C for four hours.

At the next stage, 100 microliters of dimethyl sulfoxide was added to dissolve the purple formazan crystals.

The measured absorption at 570 nanometers was recorded by a microplate reader (model: Anthos 2020, Biochrom, Germany). It is also notable that the blank samples contained no scaffolds, and all the measurements were performed in triplicate.

The cell viability rate was determined using the following equation:

Cell viability=570 OD (sample)/ 570 OD (control) × 100

Mineralization

Alizarin red stain (ARS) is a dye that selectively binds to calcium salts and was used to estimate the mineral content of the scaffolds in the current research. To this end, the scaffolds were initially stabilized in one milliliter of formaldehyde for 10 minutes and stained with one milliliter of 40 mM ARS afterwards (pH: 4.1) for 30 minutes.

Using deionized water, the scaffolds were cleaned until the unbound dye was removed. Following that, the samples were transferred to two-milliliter tubes containing 1.5 milliliters of 50% acetic acid.

The scaffolds remained in acetic acid for 18 hours at room temperature in order to ensure that all the bound dye was dissolved. At the next stage, 500 microliters of the solubilized stain was sucked up into a 1.5-milliliter tube containing 600 microliters of NaOH (1 M) to adjust the pH to 4.1. Afterwards, 200 microliters of the solution was moved to a 96-well plate. Absorbance was measured at 550 nanometers using a microplate spectrophotometer (model: SpectraMax plus 384, Molecular Device, USA).

Statistical analyses

Data analysis was performed in GraphPad Prism software version 6 (GraphPad Software Inc., USA) for the plotting of the graphs and statistical analysis.

The obtained data were expressed as mean and standard deviation, comparisons were carried out using one-way analysis of variance (ANOVA) and two-way ANOVA at the significance level of $P<0.05$. Each experiment was repeated at least three times.

RESULTS AND DISCUSSION

Morphology of the scaffolds

The morphology of the scaffolds was evaluated using SEM. As is depicted in Fig 1, the PCL scaffolds showed a well-defined internal geometry with a highly porous structure and appropriate pore interconnectivity. The prepared scaffolds had variable pore sizes, ranging from several microns to a few hundred microns, which were considered appropriate for maintaining cellular growth and vascularization [22].

Decreased cell aggregations that may develop along scaffold edges is an additional advantage for the large pore sizes obtained in the present study [23].

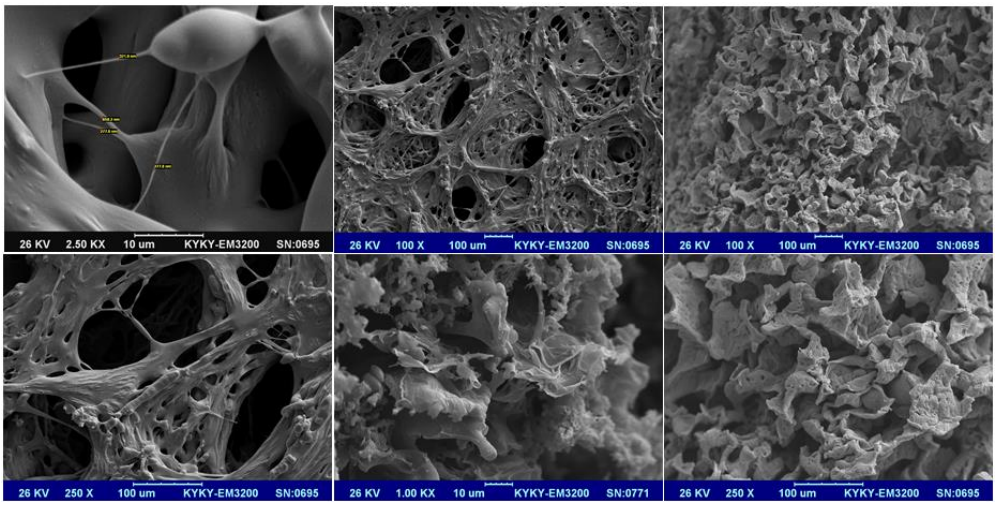

Fig 1. Scanning Electron Microscopy (SEM) Image of Engineered PCL Scaffolds without Coating

Particle size of bioactive glass 45S5 and GEL NPs

Fig 2 shows the SEM images of the BG used in the present study. Particle size was estimated using the ImageJ software (400±26.45 nm). The results of DLS regarding the GEL NPs are illustrated in Fig 3. The particle size (d50) of the GEL NPs was estimated at 234 nanometers, which is consistent with a previous report on the GEL NPs prepared by a two-step desolvation technique, with the size calculated to be 252 nanometers [24].

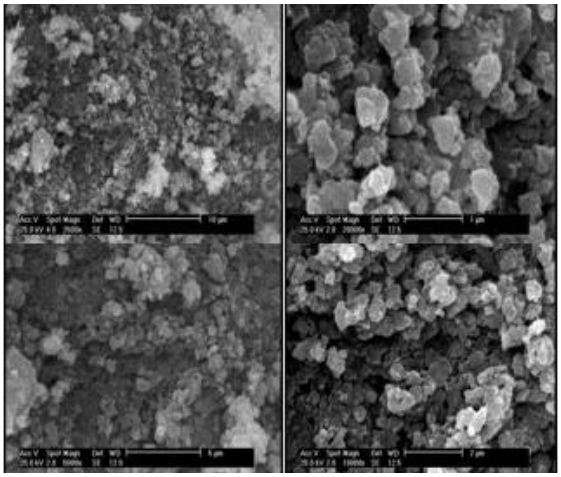

Fig 2. SEM Image of Bioactive Glasses

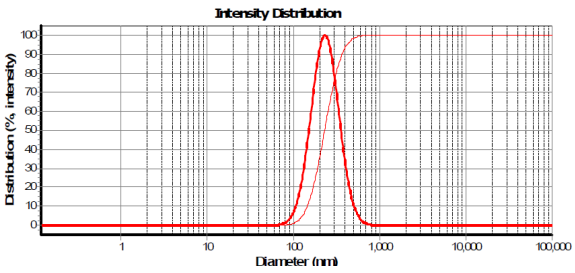

Fig 3. DLS Results of Gelatin Nanoparticles Prepared Using Desolvation Method

FTIR spectroscopy

Fig 4 shows the FTIR spectra of pure PCL and PCL scaffolds with various coatings. Several characteristic bands of PCL were observed at 2,852 cm^{-1} (symmetric -CH2 stretching), 2,921 cm^{-1} (asymmetric -CH2), 1,728 cm^{-1} (carbonyl stretching), and 1,182 cm^{-1} (symmetric C-O-C stretching). The IR spectrum of GEL exhibited characteristic bands at 3,446 cm^{-1} (N-H stretching of amide bond), 1,640 cm^{-1} (C=O stretching), and 1,543 cm^{-1} (N-H bending). Similar results have been previously reported, such as 2,949 cm^{-1} for asymmetric -CH2 stretching, 2,865 cm^{-1} for symmetric -CH2 stretching, 1,728 cm^{-1} for carbonyl stretching, and 1,170 cm^{-1} for symmetric C-O-C stretching [25, 26]. Owing to the presence of amino (NH2) and hydroxyl (OH) groups on the molecular chains of CH and GEL, these polymers are eligible for the formation of hydrogen bonds with the carbonyl groups of PCL.

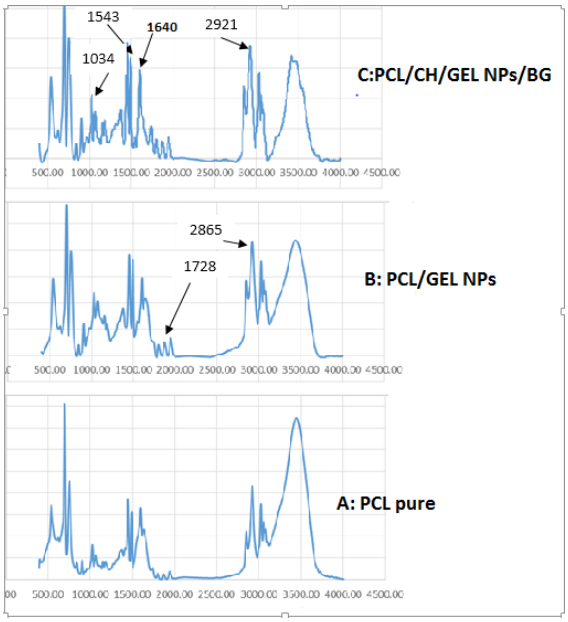

Fig 4. FTIR Analysis of A) Pure PCL, B) PCL/GEL NPs and C) PCL/CH/GEL NPs/BG

Contact angle measurement

The contact angles of the samples were measured before and after surface modification (Fig 5). Pure PCL has been observed to be highly hydrophobic (~111º) as reported previously [27]. In the current research, while the surface coating of the PCL scaffolds with CH and GEL decreased its hydrophobicity through the amino (NH2) and hydroxyl (OH) groups on their molecular chains.

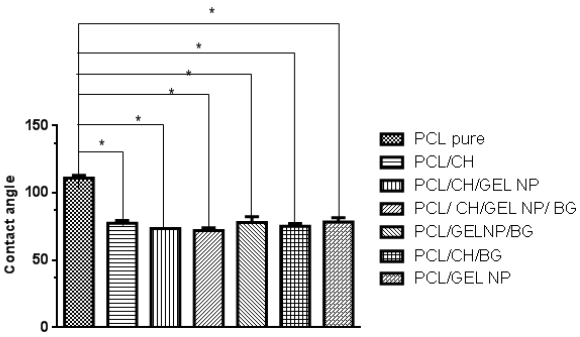

Fig 5. Water Contact Angle of Pure PCL and PCL Coated with Various Coatings (*significant differences with pure PCL group; P<0.05)

In general, the synthetic polymers have a higher contact angle compared to natural polymers [28], which is considered to be an improper factor for cell-scaffold interactions [29].

In the present study, the contact angle of pure PCL and PC/CH/GEL NPs was 128º and 80º, respectively [30]. Previous studies have elaborated on the effect of GEL as a hydrophilic polymer on the reduction of the contact angle of fibers [31].

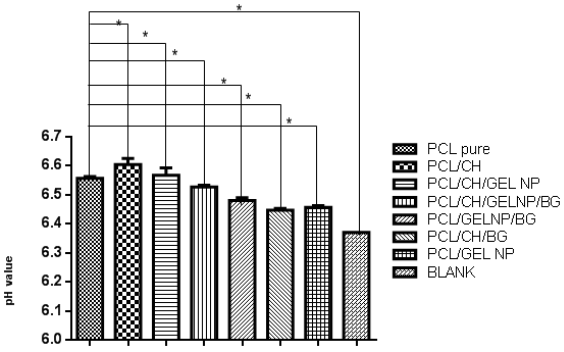

Fig 6. Measurement of pH after 1st Week Showing Effect of Various Coatings on pH Value of Solution and Significant Differences with Pure PCL Group (*significant differences; P<0.05)

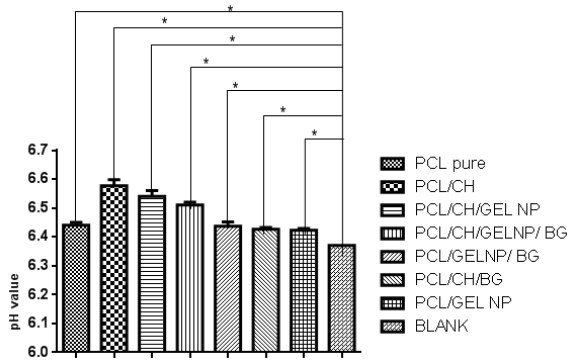

Fig 7. Measurement of pH after 2nd Week Showing Effect of Various Coatings on pH Value of Solution and Significant Differences with Pure PCL Group (*significant differences; P<0.05)

Changes in pH

In the current research, changes in pH were monitored weekly for four weeks (Figs 6-9). Furthermore, changes in the pH value of the aqueous medium were measured to assess the release of acid residues from the PCL samples. Significant pH changes cannot be tolerated by cells, thereby causing cytotoxicity. The present study was performed in the absence of cells to prevent possible changes in pH as a function of cell growth [32].

According to the obtained results, there was small, significant difference between the measured values during four weeks (P<0.05). It is also notable that the amino groups of CH could act as a buffer to slow down the rate of pH increase [33].

In general, only small changes in pH are expected. For instance, in a previous study, PCL scaffolds decreased pH from 7.27 to 7.24 within five weeks, which is in line with our findings [34].

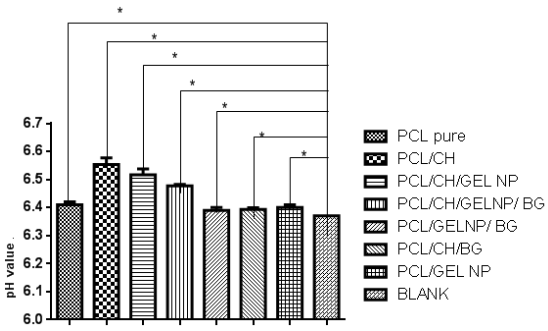

Fig 8. Measurement of pH after 3rd Week Showing Effect of Various Coatings on pH Value of Solution and Significant Differences with Pure PCL Group (*significant differences; P<0.05)

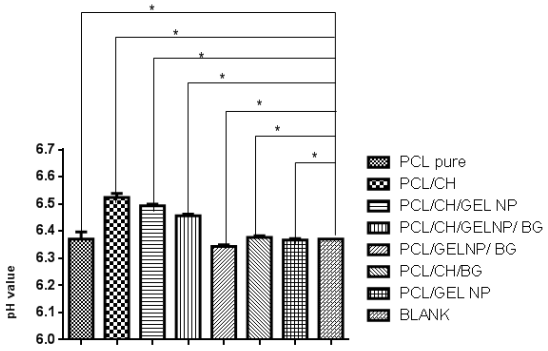

Fig 9. Measurement of pH after 4th Week Showing Effect of Various Coatings on pH Value of Solution and Significant Differences with Pure PCL Group (*significant differences; P<0.05)

Weight loss

Weight loss measurement was carried out for 60 days. During this period, the media were refreshed weakly. Every 30 days, the samples were removed from the media, rinsed with distilled water, and dried under vacuum for weight measurement (Fig 10).

According to the obtained results, there was a significant difference in weight loss as an indicator of degradation rate [35] between the modified and unmodified samples. Therefore, it could be concluded that the modified samples had significantly higher degradation rates compared to

pure PCL (P< 0.05).

According to a study regarding the weight loss of pure PCL and modified scaffold, weight loss was reported to be 12% and 23% 28 days, respectively, which is consistent with our findings [36].

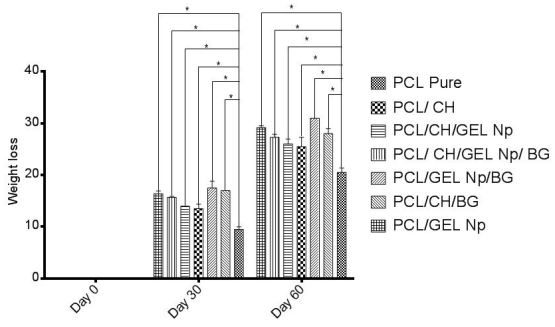

Fig 10. Results of Weight Loss Measurement on Days zero, 30, and 60 Showing Effect of Various Coatings on Scaffold Weight Loss Compared to Pure PCL (*significant differences; P<0.05)

Porosity

In the present study, porosity was examined using the liquid displacement method (Fig 11). The obtained results indicated the porosity of >70% in all the samples, which was adequate to maintain cell growth and metabolite diffusion in the bone marrow stromal cells [37, 38]. Moreover, a significant difference (up to 10%) was observed between the porosity of pure PCL and the modified samples (P<0.05). In another research in this regard, CH was reported to decrease the porosity of PCL scaffolds by approximately 6% at the CH:PCL ratio of 75: 25 [39].

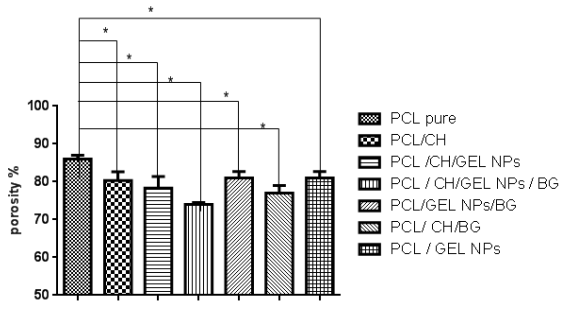

Fig 11. Porosity of PCL Scaffolds with and without Various Coatings Showing Significant Differences with Pure PCL Group (*significant differences; P<0.05)

Mechanical properties

Compressive test is a common method to evaluate the mechanical strength of scaffolds. In this approach, the sample is subjected to an increasing load until it is crushed. As is shown in Fig 12, the scaffolds produced in the present study possessed sufficient mechanical properties for tissue engineering applications. In addition, the mechanical strength of the modified scaffolds was higher compared to pure PCL. Nevertheless, all the obtained mechanical strength values were significantly higher than 2 MPa, which is the minimum value required for the bone tissues [40].

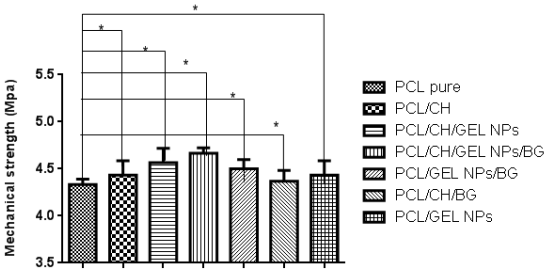

Fig 12. Mechanical Strength of Scaffolds as a Function of Various Coatings Showing Significant Differences with Pure PCL Group (*significant differences; P<0.05)

Cell viability

For tissue engineering applications, scaffolds should meet various criteria, including biocompatibility, biodegradability, and ability to harbor cells [41]. To examine the biocompatibility of the scaffolds in the present study, we used the MTT assay (Figs 13 & 14). A significant difference was observed between the modified scaffolds and pure PCL in this regard (P<0.05).Accordingly, the scaffolds that were modified with GEL NPs and CH exhibited a relatively higher rate of cell proliferation compared to the other groups. Similar results have been reported in previous studies [33], which could be attributed to the increased hydrophilicity of modified scaffolds.

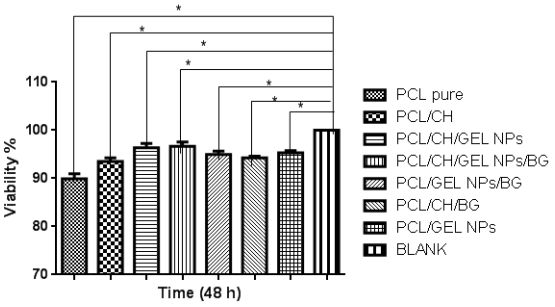

Fig 13. MG63 Cell Viability at 48 Hours as a Function of Coating Scaffolds with CH, GEL NPs, and/or Bioactive Glass Showing Significant Differences with Pure PCL Group (P<0.05)

Cells-scaffold interactions

Fig 15 shows the SEM images of the MG-63 cells cultured on the scaffolds. The cells on the pure PCL

scaffolds had almost spherical morphology with poor spreading tendency, which could be due to the high hydrophobicity of PCL. It has previously been reported that the proliferative capacity of cells may be hindered by hydrophobic scaffolds [42].

In the current research, the coating of the scaffolds resulted in improved cell adhesion and spreading compared to the pure PCL scaffolds, which could be attributed to the improved hydrophilicity of the scaffolds [33].

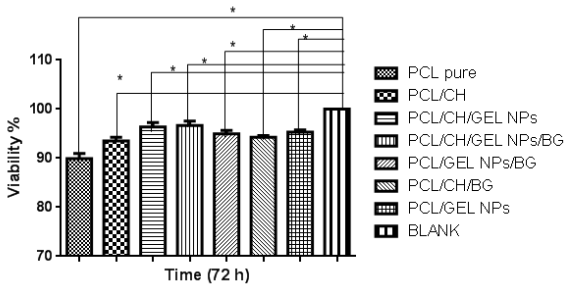

Fig 14. MG63 Cell Viability at 72 Hours as a Function of Coating Scaffolds with CH, GEL NPs, and/or Bioactive Glass (*significant differences; P<0.05)

Mineralization

Fig 16 depicts the results of alizarin red staining. A significant difference was observed in hydroxyapatite deposition between the modified samples and pure PCL samples (P<0.05).

Most calcium accumulation was detected in the MG-63 cells that were seeded on the PCL/CH/GEL NPs scaffolds with the highest mineralization value (OD: 1.126).

These findings are in line with a previous study on PLLA nanofibers modified with collagen/nano-hydroxyapatite [43].

CONCLUSION

In the present study, we fabricated nanofibrous and macroporous GEL-coated scaffolds using the TIPS technique.

TIPS demonstrated favorable control on porosity, mechanical properties, pore interconnectivity, and pore size in the three-dimensional scaffolds. Furthermore, our findings indicated that the TIPS method is a promising technique to produce scaffolds for bone tissue engineering.

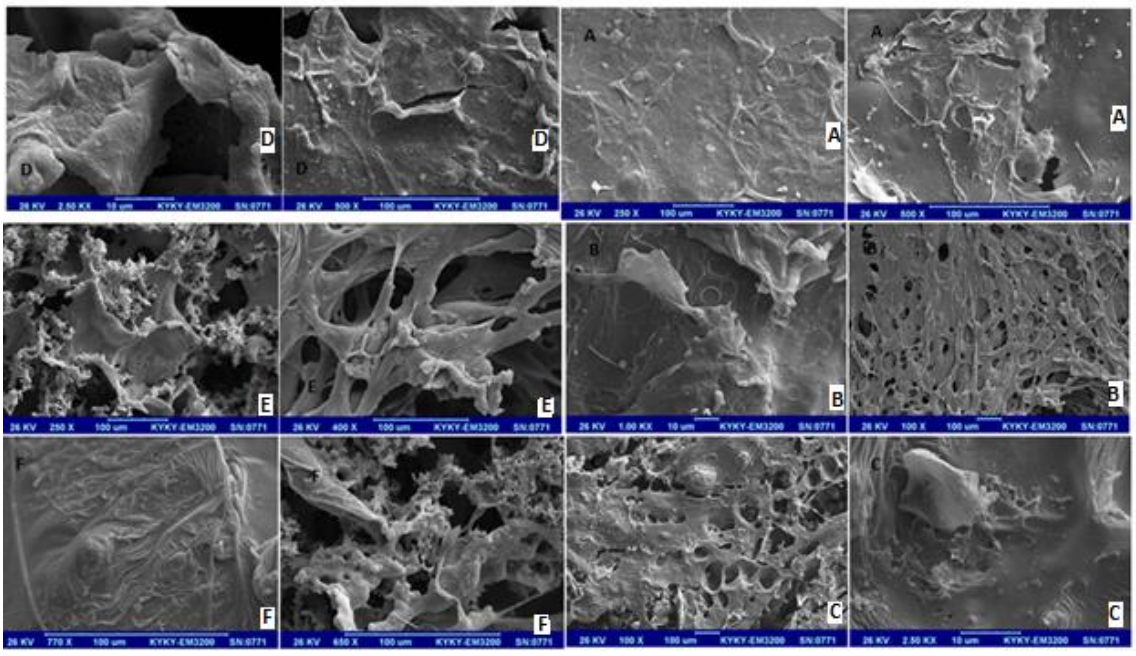

Fig 15. SEM Images of Cell Interaction Studies of MG63 Cell Line on Scaffolds with and without Coating A) PCL/CH/GEL NPs/BG, B) PCL/GEL NPs, C) PCL/GEL NPs, D) PCL/CH/BG, E) PCL/GEL NPs/BG, and F) PCL/CH

According to the results, surface functionalization improved the properties of the scaffolds, thereby enhancing cell scaffold interactions. On the other hand, surface coating with CH/GEL NPs/BG yielded better results compared to the other samples.

ACKNOWLEDGMENTS

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